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54 Plants transformed with a DNA sequence from *Bacillus thuringiensis*.

57 A plant cell transformed with a *Bacillus thuringiensis* gene coding for a protein of at least 66 kDa that is toxic to Coleoptera. A plant containing the transformed cell is resistant to Coleoptera.

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Description

PLANTS TRANSFORMED WITH A DNA SEQUENCE FROM BACILLUS THURINGIENSIS

Background of the Invention

This invention relates to a DNA sequence (the "bt13 gene") which has been found independently in the genome of both the strains *Bacillus thuringiensis tenebrionis* (U.K. patent application 87.19414) and *Bacillus thuringiensis* S1 (U.K. patent application 87.30261). The bt13 gene encodes a 66 kDa crystal protein (the "Bt13 protein") which is believed to be the active protein in the crystal toxin (the "Bt13 toxin") produced by these B.t. strains. The Bt13 toxin is toxic to Coleoptera (beetles).

This invention particularly relates to a plant, such as a tomato or potato plant, the genome of which is transformed with the bt13 gene so that some or all of the cells of the plant produce the Bt13 protein, thereby rendering the plant resistant to Coleoptera.

The bt13 gene has been found to be identical in nucleotide sequence to: 1) the B.t. gene that is disclosed in European patent publication 0,213,818 (1987) and that codes for a Coleoptera toxin containing a 86 kDa protein; and 2) the gene found in the B. t. strain which is disclosed in European patent publication 0,149,162 (1985) and which produces a Coleoptera toxin containing proteins of 20, 40, 65 and 70 kDa.

Summary of the Invention

In accordance with this invention, a plant cell genome is transformed with all part of the bt13 gene, particularly the part of the bt13 gene coding for the Bt13 protein. The resulting plant cell can be used to produce a plant, in which some or all of the plant cells express the gene by producing the Bt13 protein, thereby rendering the plant Coleoptera resistant.

Also in accordance with this invention, a method is provided for transforming the plant cell genome with all or part of the bt13 gene.

Further in accordance with this invention, a DNA sequence is provided that can be obtained from the bt13 gene and that codes just for the Bt13 protein. A plant cell, transformed with this DNA sequence can produce a Coleoptera toxin that consists essentially of the Bt13 protein.

Detailed Description of the Invention

In accordance with this invention, the bt13 gene can be isolated in a conventional manner from the B. t. strains deposited at the Deutsche Sammlung Von Mikroorganismen ("DSM") at Göttingen, Federal Republic of Germany: 1) under accession number 4288 on October 22, 1987 (as described in U.K. patent application 87.30261); and 2) under accession number 2803 (as described in European patent publication 0,149,162). In this regard, the DNA sequence of the bt13 gene can be deduced from the amino acid sequence of the Bt13 protein that is produced by the deposited B. t. strains and is toxic to Coleoptera.

The bt13 gene can also be stably inserted in a conventional manner into the nuclear genome of a single plant cell, and the so-transformed plant cell be used to produce a transformed plant in a conventional manner. In this regard, a Ti-plasmid, containing the bt13 gene, in *Agrobacterium tumefaciens* can be used to transform the plant cell, using the procedures described, for example, in European patent application 0,116,718, PCT publication WO 84/02913 and European patent application 87/400,544.0 (which are incorporated herein by reference).

Preferably, the bt13 gene is inserted in a plant genome downstream of, and under the control of, a promoter which directs the expression of the gene in the plant cell. Preferred promoters include the strong constitutive exogenous plant promoters such as: the promoter from cauliflower mosaic virus directing the 35S transcript (Odell, J.T., Nagy, J. and Chua, N.M. (1985) *Nature* 313 810-812) [the "35S promoter"]; the 35S promoter from the CaMV isolate Cabb-JI (Hull and Howell (1987) *Virology* 86, 482-493) [the "35S3 promoter"]; and the TR1' promoter and the TR2' promoter which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten et al (1984) *EMBO J.* 3, 2723-2730) [the "TR1' promoter" and "TR2' promoter", respectively]. Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (e.g., leaves and/or roots) whereby the Bt13 protein is expressed only in cells of the specific tissue(s) or organ(s). For example, the bt13 gene could be selectively expressed in the leaves of a plant (e.g., tomato or potato) by placing the gene under the control of a light-inducible promoter such as the promoter of the ribulose-1,5-biphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in U.S. patent application 821,582, filed January 22, 1986 (which is also incorporated herein by reference). Another alternative is to use a promoter whose expression is inducible, (e.g., by temperature or chemical factors).

It is further preferred that the bt13 gene be inserted in the plant genome upstream of suitable polyadenylation and transcription termination signals such as that of: the octopine synthase gene (Gielen et al. (1984) *EMBO J.* 3, 835-845); or the T-DNA gene 7 (Velten and Schell (1985) *Nucleic Acid Research* ("NAR") 13, 6981-6998).

It is further preferred that the bt13 gene be in the same transcriptional unit as, and under the control of, the same promoter as a selectable marker gene whereby the two genes are expressed in a transformed plant as a fusion protein (U.S. patent application 821,582, filed January 22, 1986; Vaeck et al (1987) Nature 327, 33-37). Any conventional marker gene can be utilized, the expression of which can be used to select transformed plant cells. An example of a suitable selectable marker gene is an antibiotic resistance gene such as the neo gene coding for kanamycin resistance (European patent application 87/400,544.0; U.S. patent application 821,582).

It is still further preferred that the bt13 gene be inserted in the plant cell genome, together with a gene, such as the bt2 gene (U.S. patent application 821,582; Höfte et al (1986) Eur. J. Biochem. 161, 273-280), which codes for a toxin against Lepidoptera. Thereby, a transformed plant can be produced which is resistant to both Lepidoptera and Coleoptera.

The following Examples illustrate the invention. The Figures referred to in the Examples, are as follows:

Fig. 1 shows the restriction map of the 2.9 kb HindIII fragment of the plasmids pBt130 and pBt130' containing the bt13 gene in Examples 1 and 2.

Fig. 2 shows the nucleotide sequence, including an open reading frame ("ORF"), of the bt13 gene and the deduced amino acid sequence of the Bt13 protein. The bt13 gene has the nucleotide sequence shown in Fig. 2 from nucleotide 566 to nucleotide 2497. A synthetic DNA probe, used for screening cosmid clones, is also indicated, and the mismatches with the bt13 gene's DNA sequence are underlined. The 22 N-terminal amino acids of the Bt13 protein are indicated. The question mark ("??") indicates that this amino acid residue was not unambiguously determined, and two possible amino acid residues are shown. The in-frame ATG-codons of the bt13 gene are underlined twice. The first in-frame upstream stopcodon is boxed, and the putative Shine-Dalgarno sequence is underlined.

Fig. 3 shows the 3' end sequence of the bt13 gene with the predicted amino acid sequence of the Bt13 protein. The homologous sequences in other known B.t. toxins are indicated. The arrows indicate the C-terminus of the largest non-toxic fragment (the "fragment 1") and the smallest toxic fragment (the "fragment 2") of the Bt2 toxin (U.S. patent application 821,582; Höfte et al (1986)). The numbering in this figure does not correspond to the numbering of the sequence shown in Fig. 2.

Fig. 4 shows schematically the cloning of the 5' end (fragment 1) and the 3' end (fragment 2) of the bt13 gene into the plasmid pMA5-8.

Fig. 5 shows schematically the mutagenesis of the 5' and the 3' end of the bt13 gene. The nucleotide sequence changes are boxed. The codons of the bt13 gene are numbered.

Fig. 6 shows schematically: A) the cloning of the modified 3' end of the bt13 gene into the plasmid pUC19 to form the vector pBt136 and the arrangement of the inserted fragment in pBt136; and B) the construction of E. coli expression vectors pBt134 and pBt135 from pBt136.

Fig. 7 shows schematically the construction of a plasmid pBt13neo of fused genes and the sequence at the junction of the fused genes.

Fig. 8 shows schematically the construction of plant expression vectors containing all or part of the bt-13 gene: A) pGSJ141 and pGSJ142; B) pGSJ143 and pGSJ144; C) pGSJ147 and pGSJ148; and D) pTVE38.

Fig. 9 shows schematically the construction of plant expression vectors PGSJ145 and pGSJ146 containing the bt13 gene.

Unless otherwise stated in the Examples, all procedures for making and manipulating recombinant DNA were carried out by the standardized procedures described in Maniatis et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982). E. coli containing the following plasmids and vectors, used in the Examples, have been deposited in the DSM under the provisions of the Budapest Treaty:

| Plasmid or vector | DSM Accession No. | Date |
|-------------------------|----------------------|----------------|
| pbt13-neo | 4752 | August 3, 1988 |
| pGSH150 | 4753 | August 3, 1988 |

Example 1 - Isolating the bt13 gene

A polyclonal antiserum was raised in mouse against total Bacillus thuringiensis tenebrionis crystal toxin solubilized in a buffer solution containing 50 mM Na₂CO₃, pH 10 + 10 mM dithiothreitol ("DTT"). The crystal toxin was purified according to Mahillon, J., and Delcour, J. (1984) J. Microbiol. Methods 3, 69-73. The crystal toxin contained one major 66 kDa protein as shown in SDS-PAGE (Laemmli, U. (1970) Nature 227, 680-685). The N-terminal sequence of the major 66 kDa protein in the crystals was determined. To this end, total crystal

proteins were blotted on polybrene coated glass fibers (European patent application 86/401933.6), and the 66 kDa protein was extracted for amino acid sequencing. The sequence of the first 22 amino acids, as shown in Fig. 2, were determined by gas phase sequencing using a gas phase sequencer (Applied Biosystems Inc. USA) operated as described by Hewick, R.M., Hunkapillar, M.W., Hood, L.E. and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990-7997. Purified solubilized crystals showed a LD50 of < 100 ng/cm² against the Colorado potato beetle (*Leptinotarsa decemlineata*).

A library of partial *Sau3A*-digested total DNA of *B. t. tenebrionis* was constructed in the cosmid vector pLAFB1 which is a derivative of pLAFR1 (Friedman, A.M., Long, S.R., Brown, S.E., Biukema, W.J. and Ansubel, F.M. (1982) Gene 18, 289-296) with a *Bam*HI-linker in the former unique *Eco*R1 site. The polyclonal mouse antiserum, raised against the crystal toxin of *B. t. tenebrionis*, was used to screen approximately 2000 clones in a colony immuno assay. Twenty-one positive clones were further analysed by Western blotting (Towbin, H., Stähelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA ("PNAS") 76, 4350-4353) of total cellular extracts. Among these clones, four different types of clones could be distinguished based upon the visualized cross-reacting proteins. The four types of clones are set forth in the following Table 1.

Table 1: types of *E. coli* clones expressing proteins that cross-react with *B.t. tenebrionis* crystal antiserum

| Type | Example of clone | Size of cross- reacting proteins (kDa) | hybridization to a synthetic oligonucleotide (29-mer) |
|------|---------------------|--|--|
| I | pBTT16 | 66 | - |
| II | pBTT18 | 66 + 71 (broad band) | - |
| III | pBTT32 | 66 + 71 (fine band) | + |
| IV | pBTT30 | larger strongly cross reacting protein | - |

A 29 bp degenerated DNA probe was synthesized based upon a sequence of 10 amino acids (Fig. 2) of the *B.t.tenebrionis* crystal toxin using the procedure of Itokaru et al (1984) Ann. Rev. of Biochem. 53, 323-356. Representatives of the four types of cosmid clones were hybridized at low stringency reaction conditions.

Only pBTT32 (Type III) showed homology with the probe. The hybridizing sequence was localized on a 1.6 kb *Eco*RI- *Hind*III fragment. DNA sequencing (Maxam and Gilbert (1980) Methods In Enzymology 65, 499-560) from both sides of the fragment revealed that an open reading frame, showing some homology to the *bt2* gene (Höfte et al (1986), could be localized on a 2.9 kb *Hind*III fragment. This fragment was subcloned in pUC8 (Vieira, J. and Messing, J. (1982) Gene 19, 259-268) giving rise to plasmid pBt130 (Fig. 4).

pBt130 directed the synthesis in *E. coli* of a 71 kDa protein which reacted with the polyclonal mouse anti-*B.t. tenebrionis* crystal toxin serum as shown in a Western blot. This 71 kDa protein was processed in *E. coli* into a protein of the same size as the major 66 kDa crystal protein of *B. t. tenebrionis*. pBt130 contained the *bt13* gene in the opposite orientation with respect to the promoter (pLac) of the vector. Therefore, the gene was transcribed from its own promoter. To enhance its expression, the *bt13* gene was cloned, as a *Hind*III fragment behind the lambda P_L-promoter in plasmid pLK59 (Botterman, J. and Zabeau, M. (1987) DNA 6, 583-591) which was then used to transform the *E. coli* strain K-12ΔH1Δtrp carrying a temperature sensitive repressor of the lambda P_L promoter (Bernard et al (1979) Gene 5, 59-76). This construction provided a high level of expression of the Bt13 protein upon temperature induction at 42°C of the transformed *E. coli* K-12ΔH1Δtrp.

Example 2 - Isolating another *bt13* gene (the "bt13' gene")

The crystal toxin of the *B.t.* strain, deposited at the DSM under accession no. 4288, was purified using the procedure described by Krieg, V.A., Huger, A.M., Langenbruch, G.A. and Schnetter, W. (1983) Z. Angew. Entomology 96, 500-508. A polyclonal antiserum was raised in rabbit (U.S. patent application 821,582) against the crystal toxin produced by the *B.t.* strain DSM 4288. This antiserum was used to detect the crystal protein produced by this *B.t.* strain by Western blotting.

The 66 kDa crystal protein was shown by SDS-page (Laemmli, U. (1970)) to be the major component of the

crystal toxin produced by the *B.t.* strain DSM 4288. The N-terminal sequence of the 66 kDa protein in the toxin was then determined. To this end, total crystal proteins were blotted on polybrene-coated glass fibers (European patent application 86/401933.6), and an approximately 66 kDa fragment was extracted for amino acid sequencing by gas phase sequencing. (Hewick et al (1981)).

A library of partial Sau3A-digested total DNA of the strain was constructed in the plasmid pUC8 (Viera and Messing (1982)) using the BamHI site. An approximately 1000 basepairs BalI-XbaI fragment (Fig. 1) derived from the bt13 gene of Example 1 was radioactively (³²P) labelled and used as a probe to screen the library. Plasmid DNA was extracted from strongly hybridizing clones, digested with HindIII and analyzed in Southern blotting with the probe derived from the bt13 gene. Several plasmids were identified, containing a 2.9 kb HindIII fragment that hybridized with the probe. DNA sequencing (Maxam and Gilbert (1980)) from both sides of the HindIII fragment revealed that an ORF, showing homology to the bt13 gene of Example 1, was localized on this HindIII fragment. This fragment was subcloned in pUC8 (Viera and Messing (1982)), giving rise to plasmid pBt130'.

pBt130' directed the synthesis in *E. coli* of a 71 kDa protein which reacted with the polyclonal rabbit anti-serum to the crystal toxin of *B.t.* strain DSM 4288 in Western blot. The 71 kDa protein was processed in *E. coli* into a protein of the same size as the major 66 kDa crystal protein of *B.t.* strain DSM 4288. pBt130' contained the bt13' gene in the opposite orientation with respect to the promoter (pLac) of the vector. Therefore, the gene was transcribed from its own promoter. To enhance the expression thereof, the bt13' gene was cloned as a HindIII-fragment behind the lambda P_L-promoter in plasmid pLK59 (Botterman and Zabeau (1987)), which was then used to transform the *E. coli* strain K-12ΔH1Δtrp (Bernard et al (1979)). This construction provided a high level of expression of the bt13' gene upon temperature induction at 42°C of the transformed *E. coli* K-12ΔH1Δtrp.

Example 3 - Comparing of the structure of the bt13 gene and the bt13' gene

It was found that the bt13 gene of Example 1 and the bt13' gene of Example 2 are identical despite the fact that they were independently isolated from two strains discovered in different areas. Both bt13 genes encode a polypeptide of 644 amino acids with a predicted molecular weight of 71 kDa. The ATG Initiator codon in both bt13 genes is localized at position 566 in the DNA sequence of the genes (Fig. 2). This conclusion was based upon the following assumptions:

A) The N-terminal sequence of the major 66 kDa crystal protein coincides with the predicted amino acid sequence from both bt13 genes starting at position 737 (Fig. 2). This indicates that the 66 kDa protein is a processed product of a longer 71 kDa precursor. Both the 66 and 71 kDa proteins were also observed in the *E. coli* clones expressing the bt13 gene in Examples 1 and 2.

B) The first in-frame stopcodon upstream of bp 737 is at position 527. In between, there are three in-frame ATG-codons. The ATG at position 566 is a very likely candidate for an initiator codon:

1) It is the only in-frame ATG codon preceded by a clear Shine and Dalgarno sequence (Shine, J. and Dalgarno, L. (1974) PNAS 71, 1342-1346).

2) The predicted N-terminal sequence starting at this position shows homology to the N-terminus of the bt13 gene: M - - N P N N
the bt2 gene: M D N N P N

C) The ORF, starting at position 566, ends at position 2497.

D) The 66kDa proteins, coded by both bt13 genes, show (in Fig. 3) local stretches of homology with the Bt2 and Bt8 crystal toxins produced by the *B.t.* berliner 1715 strain (Höfte et al (1986)) and the *B. t.* israelensis 4Q-272 strain (US application serial No. 021,405, filed March 4, 1987; Chungjatupornchai et al (1988) Eur. J. Biochem. 173, 9-16), respectively. The C-terminal sequence of each of the bt13 genes also shows a strong homology with known sequences in other *B.t.* toxins (Fig. 3). Moreover, the C-terminus of the smallest toxic fragment of the Bt2 crystal toxin (Vaeck et al (1987)), as determined by deletion mapping, coincides with the amino acid sequence preceding the C-terminal amino acid of the crystal proteins of each of the bt13 genes. The Bt13 protein can thus be considered as a naturally occurring truncated active toxin.

These assumptions have been confirmed by McPherson et al (1988) Bio/Technology 6, 61-66.

Example 4 - Producing Bt13 protein in *E.coli*

Using the procedure of Höfte et al (1986), a purified Bt13 protein (66 kDa), together with its 71 kDa precursor, was produced from induced cultures of the transformed *E. coli* K-12ΔH1Δtrp of Examples 1 and 2.

The Bt13 protein, so produced, showed an LD50 of 12 ng/cm² against *Leptinotarsa decemlineata*.

Example 5 - Constructing plant transformation cassettes with the bt13 gene

In order to obtain optimal expression of the bt13 gene in transgenic plants, chimeric constructs were made as shown in Figs. 4 to 9 with different strong transcription initiation signals (promoters), using each of the bt13 genes of Examples 1 and 2. Since the bt13 gene in the *E. coli* K-12ΔH1Δtrp strain of Examples 1 and 2 was not readily available as a gene cassette, the gene was modified as follows.

A BamHI site was created abutting the ATG codon of the bt13 gene. The 4th nucleotide of the bt13 gene was

changed from an A to a G by site directed mutagenesis yielding an unique BamHI site in the gene by the procedure described in the article by Stanssens, P., McKeown, Y., Friedrich, K. and Fritz, H.J. (1987), entitled "Oligonucleotide-directed construction of mutations by the gapped duplex DNA method using the pMa/c phasmid vectors", published in the Collection of experimental procedures distributed at the EMBO course entitled "Directed mutagenesis and protein engineering" in July 1987 at the Max Planck Institut für Biochemie, Martinsried, Federal Republic of Germany. According to the "Kozak-rules" (Kozak, M. (1986) Cell 44, 283-292), this mutation should also optimize the translation initiation in plant cells. However, this mutation also changes the 2nd amino acid of the bt13 gene from Asn to Asp. The effect of this mutation on the activity of the Bt13 protein was assessed in a toxicity assay after expression of the modified protein in E. coli.

In order to avoid a change of the second codon of the bt13 gene, an alternative construction was made. A HinfI site was provided directly downstream of the ATG-initiator codon ('AT G AAT C C') to permit the direct fusion of the second codon to the initiator present in plant promoter fragments.

Further constructions carrying the fragment encoding the processed 66 kDa protein were made. It is believed that the Bt13 protein is synthesized as a precursor of about 71 kDa that is processed into the 66 kDa Bt13 protein in E. coli and in B.t. strains (during the crystal formation). Unlike other B.t. toxins (Höfte et al (August 1988) Applied and Environmental Microbiology, In press), the 66 kDa Bt13 protein is present in the processed form already in the crystals of B. thuringiensis tenebrionis. Because it is unknown if the 71 kDa precursor protein would be correctly activated in the midgut of Coleoptera larvae or in plant cells, an alternative approach was to express the gene fragment encoding the processed 66 kDa protein, as such, in transformed plants. To this end, a restriction site was created at the putative processing site of the amino acid at position 58 of the precursor protein (Fig. 2).

A FokI site was introduced 9 basepairs upstream of the first codon of the gene fragment encoding the 66kDa protein by the mutagenesis procedure of Stanssens et al (1987). This yielded a modified bt13 gene called "bt13-57". The recognition sequence of FokI was separated from its cleavage site which allowed it to generate restriction fragments exactly at the processing site.

To allow proper fusion of the bt13 gene coding region to transcription termination and polyadenylation signals in plant expression vectors, the non-coding sequence downstream (3' end) in the bt13 gene was removed. To this end, an oligonucleotide with an EcoRI site, overlapping the TAA stopcodon, was introduced into the bt13 gene by the mutagenesis procedure of Stanssens et al (1987).

In order to be able to select easily transgenic plants producing sufficient Bt13 protein to kill Coleoptera, hybrid gene constructions were made with a gene encoding a selectable marker as described in U.S. patent application 821,582 and Vaeck et al (1987). Such a hybrid construction allows the selection of transformants whose marker gene expression is sufficiently great that one can suitably predict that the bt13 gene expression also is sufficiently great to render the transformants Coleoptera resistant (Höfte et al (1988) FEBS Letters 226, 364-370). For this purpose, bt13-neo hybrid gene fusions were constructed. The oligonucleotide with the EcoRI site, from the preceding paragraph, was used to generate a HpaI site in front of the stopcodon. This site allowed the codon 643 of the bt13 gene and of the bt13-57 gene to be fused to a neo gene fragment encoding an active neomycin phosphotransferase enzyme II ("NPTII") as described in U.S. patent application 821,582. The C-terminal Asn was not believed to be essential for toxicity. The resulting fusion proteins were produced in E. coli transformed with the bt13-neo hybrid gene fusions by the procedure described by Höfte et al (1986) and tested for Bt13 and NPTII activity as described below.

To evaluate the effect of the modifications, described above, on the activity of the Bt13 protein, the modified bt13 genes were expressed in E. coli and their modified Bt13 protein products were tested against the Colorado potato beetle. To this end, the bt13 gene fragments were fused in phase to the ATG-initiator codon of the cro gene of phage lambda under control of the P_R promoter of phage lambda (Botterman and Zabeau (1987)).

As described below, the expression of the bt13 genes in plants was obtained by placing the different bt13 cassettes, described above, under the control of a 35S, a TR1' or a TR2' promoter at the 5' end. Polyadenylation and transcription termination signals from the octopine synthase gene ("ocs") (Gielen et al (1984)) or from the T-DNA gene 7 ("g7") (Velten and Schell (1985)) were also provided at the 3' end of the gene cassettes. Except for the hybrid gene constructions, the cassettes further contained a selection marker for transformants, i.e., the neo gene, controlled by its own TR1' promoter or promoter from the nopaline synthase gene ("Pnos" -- U.S. patent application 821,582) and its own OCS polyadenylation and transcription termination signals. To generate plants protected against both Lepidoptera and Coleoptera, constructs containing both the bt2 and bt13 genes were also made. Each of these chimeric constructs was inserted in a plant transformation vector of a disarmed Ti plasmid derived from Agrobacterium tumefaciens (Deblaere et al (1985) NAR 13, 4777-4788). The plant expression constructs, that were made, are summarized in the following Table 2.

Table 2 : Plant expression constructs containing a bt13 gene

| <u>5' end</u> | <u>Bt gene</u> | <u>3' end</u> | <u>Chimeric selectable marker gene</u> | <u>Plant Expression Construct</u> | |
|---------------|----------------|---------------|--|---|----|
| TR2' | btneo860 | g7 | | pGSH152 | 10 |
| TR2' | bt884 | g7 | | pGSH163 | |
| 35S | bt13 | g7 | Pnos-neo-3'ocs | pGSJ141 | 15 |
| TR2' | bt13 | g7 | TR1'-neo-3'ocs | pGSJ142 | |
| 35S | bt13-57 | g7 | Pnos-neo-3'ocs | pGSJ143 | |
| TR2' | bt13-57 | g7 | TR1'-neo-3'ocs | pGSJ144 | 20 |
| TR1' | bt13 | ocs | | pGSJ145 | |
| 35S | bt13 | ocs | TR1'-neo-3'ocs | pGSJ146 | |
| TR2' | bt13-neo | g7 | | pGSJ147 | 25 |
| 35S | bt13-neo | g7 | | pGSJ148 | |
| 35S3 | bt13-neo | g7 | | pTVE38 | |
| 35S3 | bt13-57-neo | g7 | | pTVE39 | 30 |

For the construction of the bt13 gene cassettes, the restriction sites - BamHI, FokI, HpaI and EcoRI - were created in the bt13 gene coding region using site-directed mutagenesis with oligonucleotides as mutagenic primers (Stanssens et al (1987)). The vectors described by Stanssens et al (1987) were used. In this regard, the EcoRI-HindIII fragments respectively containing the 5' end (fragment 1) and 3' end (fragment 2) of the bt13 gene were isolated from pBt130 and cloned into pMA5-8 (On deposit at the DSM under accession number 4567), giving rise to pMABt131 and pMABt135, respectively (Fig. 4). Three oligonucleotides were then synthesized : 1) 21-mer : 5'-GGAAGAAAAAT GGATCC AAC-3'; 2) 25-mer: 5'-GCAGATAATA GGAAG CAATCAG-3'; and 3) 34-mer: 5'-CTTCTAGT GAATTC A GTTAA CTGGAATAAATTC; using the procedure described by Itekari et al (1984). Using the gapped duplex procedure of Stanssens et al (1987), oligonucleotides 1) and 2) were used to introduce respectively a BamHI and FokI site in the N-terminal sequences of the bt13 gene, and oligonucleotide 3) was used to create a HpaI and EcoRI site at the C-terminus of the gene. This yielded the respective plasmids pMCBt131, pMCBt133 and pMCBt136 (Fig. 5).

To facilitate the reconstruction of the bt13 gene, fragments from pMCBt136 and pBt130 were cloned in pUC19 (Yanish-Perron, C., Viera, J. and Messing, J. (1985) Gene 33, 103-119), yielding pBt136 as shown in Fig. 6A. In this way a unique BamHI site was introduced immediately downstream of the gene, and the fragment, containing the C-terminal end of the bt13 gene, could be used for reconstruction of the entire bt13 gene carrying the described mutations at the N-terminus.

As shown in Fig. 6B, the N-terminal fragments from the bt13 gene were retrieved from pMCBt133 as a FokI-OxaNI fragment and from pMCBt131 as a BamHI-PstI fragment. These N-terminal fragments and suitable C-terminal fragments from pBt136 were ligated in the E. coli expression vectors pJB66 and pJB63, respectively (Botterman and Zabeau (1987)), each carrying a bacteriophage lambda P_R promoter and the ribosome binding site of the cro gene of phage lambda. This yielded pBt134 and pBt135, respectively.

Making use of the HpaI site present at the 3' end of the bt13 gene in pBt135, the bt13 gene was fused in phase to a truncated neo gene encoding an active C-terminal fragment (Reiss, B., Sprengel, R., Will, H. and Schaller, H. (1984) Gene 30, 217-223). The neo gene fragment was obtained from pLKM91 (Botterman (1986)), yielding pBt13-neo as shown in Fig.7.

The plasmids pBt134, pBt135 and pBt13-neo, carrying a regulatable strong P_R promoter, were used to transform E. coli strain K-12ΔH₁Δtrp. The transformed E. coli was then temperature induced to drive expression of the different gene products as described by Botterman and Zabeau (1987). The impact of the gene mutations upon the activity of the gene products was analysed. The Bt13-NPTII fusion proteins showed

NPTII activity without adversely affecting the LD50 activity that was observed above with the intact Bt13 protein.

From the chimeric *E. coli* expression constructs pBt134, pBt135 and pBt13-neo, the *bt13* gene was isolated and inserted in plant expression vectors. Cloning of the *Bam*HI fragment from pBt135 in pGSH160 and pGSJ280 (Deblaere et al (1987) *Methods in Enzymology* 153, 277-292) yielded pGSJ141 and pGSJ142 respectively (Fig. 8A). These plasmids carried the *bt13* gene under the control of the 35S promoter (Odell et al (1985)) and the TR2' promoter (Velten and Schell (1985)), respectively. A kanamycin resistance gene was present as a selection marker for transformation. Similarly, a *Nco*I-*Bam*HI fragment from pBt134 was isolated and was ligated in the same vectors, yielding the constructs pGSJ143 and pGSJ144 containing the *bt13-57* gene under the control of the 35S and TR2' promoters respectively (Fig. 8B). Also, the *Bam*HI-*Bgl*II fragment from pBt130-neo, comprising the *bt13-neo* hybrid gene, was cloned in pGSH150 and pGSJ270 (Deblaere et al (1987)), yielding pGSJ147 and pGSJ148 with the *bt13-neo* hybrid gene under the control of the TR2' or 35S promoter (Fig. 8C). In the latter plasmids, no selection marker gene was present, since transformed plants could be directly selected for expression of the fusion protein.

In a similar way (Fig. 8D), the *bt13-neo* hybrid gene was placed under the control of a 35S3 promoter (Hull and Howell (1987)). The 35S3 promoter was cloned in pUC18 (Yanish-Perron et al (1985)), yielding pDE9, which contained an *Nco*I site at the first ATG codon in the 35S3 transcript. The 3' terminal-*Nco*I site of the promoter fragment was created at the first ATG codon by site directed mutagenesis (Stanssens et al (1987)). A *Nco*I fragment containing the *bt13* gene fragment and part of the *neo* gene was isolated from pBt13-neo and cloned in the *Nco*I site of pDE9, yielding pVE37. From pVE37, a *Hind*III-*Nar*I fragment containing the 35S3 promoter-*bt13-neo* chimeric construct was isolated and cloned in pGSH152 digested with *Hind*III and *Nar*I. This yielded pTVE38, which contained the *bt13-neo* hybrid gene under the control of the 35S3 promoter and was followed by the 3' untranslated end of the T-DNA gene 7. This chimeric construct was located between the T-DNA border sequences.

To express the *bt13* and *bt2* genes simultaneously in plants, each gene was placed under the control of its own strong constitutive promoter (Fig. 9). In this regard, a *Cla*I-*Sna*BI fragment was isolated from pGSJ141 and introduced in pGSH152 (U.S. patent application 821,582; Vaeck et al (1987)). This yielded pGSJ145 carrying the *bt13* gene under the control of the TR1' promoter (Velten and Schell (1985)) and the *bt-neo-860* hybrid gene under the control of the TR2' promoter (Velten and Schell (1985)). The *bt-neo-860* gene is a fusion of the *bt2* gene and the *neo* gene described in Example 9 of U.S. patent application 821,582.

Insertion of a *Bgl*II-*Sna*BI fragment from pGSJ142 into pGSH163 (U.S. patent application 821,582; Vaeck et al (1987)) yielded pGSJ146. pGSJ146 contained the *bt13* gene under the control of a TR2' promoter, the *bt884* gene under the control of another TR2' promoter, and the *neo* gene, as a selection marker, under the control of a TR1' promoter. The *bt884* gene is a truncated *bt2* gene as described in Example 8 of U.S. patent application 821,582.

To form a hybrid of the *bt13-57* gene, coding for just the Bt13 protein, and the *neo* gene (the "*bt-57-neo* gene") that can be expressed in plants, a plant expression construction pTVE39 was also made, using the same procedures as described above for constructing pTVE38.

Example 6 - Transformation of tobacco, potato and tomato plants

The plant expression constructs from Example 5 were transferred from *E. coli* into *Agrobacterium tumefaciens* strain C58C1Rif carrying the plasmid pGV2260 (Vaeck et al (1987); De Blaere et al (1985) *NAR* 13, 4777 et seq). The pGV2260 provides the *vir* gene functions required for transfer of the T-DNA region to the plant genome.

Mobilization of the plant expression constructs was carried out using *E. coli* HB101 containing pRK2013 (Figurski et al (1979) *PNAS* 76, 1648 et seq) as a helper (European patent publication 0,116,718). The resulting recombinant *Agrobacterium* strains were then used to transform potato plants (*Solanum tuberosum* cv. Berolina, Bintje and Désirée) by means of tuber disc infection (De Block et al (1987) *EMBO J.* 6, 2513-2518). Calli were selected on 50 mg/l kanamycin, and resistant calli were used for shoot induction. Elongated shoots were separated and transferred to rooting medium. Rooted shoots were further propagated to regenerate plants. Total DNA, prepared from cells of these plants (Dellaporta et al (1984) *Plant Molecular Biology Reporter* 1, 19-21), showed the presence of the chimeric genes by hybridization with a labelled *bt13* gene fragment.

Tobacco and tomato plants were similarly transformed with *Agrobacterium tumefaciens* and selected on kanamycin according to the methods described in European patent application 87/400141.5.

Example 7 - Expression of the *bt13-neo* and *bt13-57-neo* hybrid genes in transformed potato and tobacco plants

Calli of the potato and tobacco plants from Example 6, transformed with pTVE38 and pTVE39, were tested for NPTII activity by *in situ* phosphorylation assay using ³²P-ATP (Reiss et al (1984) *Gene* 30, 217-223; U.S. patent application 821,582). Significant NPTII activity was detected in calli of the transformed plants. Previously, the transformed calli of tobacco and potato plants had been selected on a medium containing 50 mg/l kanamycin.

These tests demonstrated that the cells of the transformed calli expressed the hybrid genes, with which

they had been transformed. Such expression involved expression of the full bt13 gene and the bt-57 gene coding for just the Bt13 protein.

Example 8 - Effect of the expression of the bt13 gene and fragments of the bt13 gene in transformed potato and tomato plants on *Leptinotarsa decemlineata* (Colorado potato beetle)

Larvae of *L. decemlineata* are fed with either potato leaves or tomato leaves in plastic containers. After 25 days, the larvae are transferred to containers filled with wet sand. The larvae dig in the sand and pupate. A week later, the adults emerge. They are placed in rearing cages with plant leaves. The eggs, laid on the leaves, are collected daily. After 4 to 5 days, the eggs hatch. The entire life cycle of the beetles occurs in a controlled environment cupboard maintained at 25°C, 70% relative humidity, and 18:6 Light: Dark photoperiod.

The killing of neonate larvae shows the toxicity of the Bt13 protein. Each larva is kept separately on a 2% agar medium and is supplied with a disc (0.28 cm²) that is cut from either:

1) a fresh tomato or potato leaf to which is applied 5 µl of an aqueous solution of the Bt13 protein from the transformed *E. coli* K-12ΔH1Δtrp of Examples 1 and 2; or

2) a fresh leaf from a transformed tomato or potato plant of Example 6. After the leaf disc is consumed, fresh pieces of untreated leaf from untransformed tomato or potato plants are supplied for the next five days. Significantly more larvae are found dead over the five days of the test among the larvae who consume the treated leaf discs and the leaf discs from transformed plants than among the control larvae who consume only untreated leaves from untransformed plants.

Needless to say, this invention is not limited to the use of the bt13 gene. Rather, the invention also encompasses the use of the DNA sequence of Fig. 2, starting at position 737 and ending at position 2497, which codes for the pure Bt13 protein, as well as other DNA sequences encoding proteins that are the same as or equivalent to the Bt13 protein. The invention further relates to all DNA recombinants including the bt13 gene or a fragment thereof suitable for the transformation of a plant cell.

This invention also is not limited to transgenic potato and tomato plants that express the bt13 gene and are resistant to Coleoptera. It also relates to any plant which can be transformed in a like manner such as oilseed rape, alfalfa, sunflower, cotton, celery, onion, cloves, corn, soybean, tobacco, brassicas and sugarbeet.

Claims

1. A transformed plant cell, characterized by a bt13 gene or a fragment thereof that codes for at least a 66 kDa protein and that is inserted in the genome of the cell.

2. The cell of claim 1, in which the bt13 gene fragment is inserted in the genome; the fragment having a DNA sequence as shown in Fig. 2, starting from position 737 and ending at position 2497 and coding for a Coleoptera toxin that consists essentially of a 66 kDa protein.

3. The cell of claim 1 or 2, in which the bt13 gene is downstream of, and under the control of, a strong constitutive plant promoter which is a 35S promoter, a TR1' promoter, or a TR2' promoter.

4. The cell of anyone of claims 1 to 3, in which the bt13 gene is upstream of polyadenylation and transcription termination signals which are from the octopine synthase gene or the T-DNA gene 7.

5. The cell of anyone of claims 1 to 4, in which the bt13 gene is in the same transcriptional unit as, and under the control of the same promoter as, a selectable marker gene.

6. The cell of anyone of claims 1 to 5 in which a gene, which codes for a Lepidoptera toxin, is also inserted in the genome of the cell.

7. A plant in which is the transformed cell of anyone of claims 1 to 6.

8. A seed of a plant of claim 7.

9. A method for rendering a plant resistant to Coleoptera characterized by: providing the plant with the transformed cell of anyone of claims 1 to 6.

10. DNA characterized by the DNA sequence shown in Figure 2, starting from position 737 and ending at position 2497.

11. A 66 kDa crystal protein encoded by the DNA sequence of claim 10.

12. A microorganism, particularly *E. coli*, transformed with the DNA sequence of claim 10.

FIG. 1

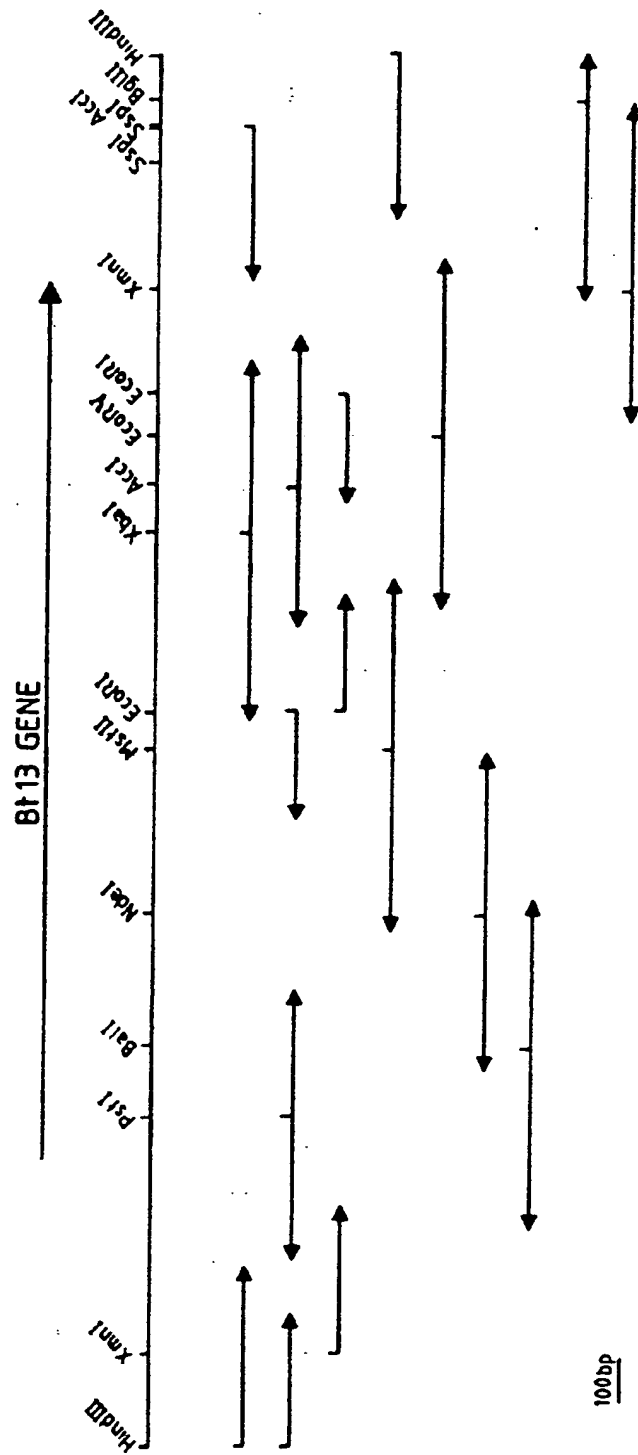


FIG 2

CTTAATTAAAGATAATATCTTTGAATTGTAACGCCCTCAAAAGTAAGAAGTACAAAAAAGAATACGTTATATAGAAATATGTTTGAAC
 10 20 30 40 50 60 70 80 90

CTTCTTCAGATTACAAATATATTCGACGGACTCTACCTCAAATGCTTATCTAACTATAGAATGACATACAAGCACAACCTTGAAAATTT
 100 110 120 130 140 150 160 170 180

GAAATATAACTACCAATGAAGTTGTTTCATGTGAATTATCGCTGATTTAATTTTCTCAATTCATATATAATATGCCAATACATTGTTA
 190 200 210 220 230 240 250 260 270

CAAGTAGAAATTAAGACACCCTTGATAGCCTTACTATACCTAACATGATGTAGTATTAAATGAATATGTAAATATATTTATGATAAGAAG
 280 290 300 310 320 330 340 350 360

CGACTTATTTATAATCATTACATATTTTCTATTGGAATGATTAAGATTCCAATAGAATAGTGTATAAATTATTTATCTTGAAAGGAGGG
 370 380 390 400 410 420 430 440 450

ATGCCATAAAAAAGGAACATTAAAAACATATATTTGCACCGTCTAATGGATTATGAAAAATCATTATATCAGTTTGAATAATTATGTAT
 460 470 480 490 500 510 520 530 540

MetAsnProAsnAsnArgSerGluHisAspThrIleLysThrThrGluAsnAsnGluValProThr
 TATGATAAGAAAGGAGGAAGAAAAATGATCCGAACAATCGAAGTGAACATGATACAATAAACTACTGAAAATAATGAGGTGCCAAC
 550 560 570 580 590 600 610 620 630

AsnHisValGlnTyrProLeuAlaGluThrProAsnProThrLeuGluAspLeuAsnTyrLysGluPheLeuArgMetThrAlaAspAsn
 TAACCATGTTCAATATCCTTTAGCGGAACTCCAAATCCAACTAGAAGATTTAAATTATAAGAGTTTTTAAGATGACTGCAGATAA
 640 650 660 670 680 690 700 710 720

AsnThrGluAlaLeuAspSerSerThrThrLysAspValIleGlnLysGlyIleSerValValGlyAspLeuLeuGlyValValGlyPhe
 TAATACGGAAGCACTAGATAGCTCTACAACAAAGATGTCATTCAAAAAGGCATTTCCGTAGTAGGTGATCTCCTAGGCGTAGTAGTTT
 730 SerSerProThrAspLysGlnValIleGlnLysGlyIleSerValValGlyAspLeuLeuGlyVal 810
 Asp Ser ? Asp
 5'- CCLACIGATAAAGACGTIATICAAAAAGG - 3'

ProPheGlyGlyAlaLeuValSerPheTyrThrAsnPheLeuAsnThrIleTrpProSerGluAspProTrpLysAlaPheMetGluGln
 CCCGTTTGGTGGAGCGCTTGTTTCGTTTATACAAACTTTTAAATACTATTTCGCCAAGTGAAGACCGTGAAGCGCTTTTATGGAACA
 820 830 840 850 860 870 880 890 900

ValGluAlaLeuMetAspGlnLysIleAlaAspTyrAlaLysAsnLysAlaLeuAlaGluLeuGlnGlyLeuGlnAsnAsnValGluAsp
 AGTAGAAGCATTGATGATCAGAAAAATAGCTGATTATGCAAAAAATAAAGCTCTTGCAGAGTTACAGGGCCTTCAAAATAATGTGAAGA
 910 920 930 940 950 960 970 980 990

TyrValSerAlaLeuSerSerTrpGlnLysAsnProValSerSerArgAsnProHisSerGlnGlyArgIleArgGluLeuPheSerGln
 TTATGTGAGTGCATTGAGTTTCATGGCAAAAAATCCTGTGAGTTCAGAAATCCACATAGCCAGGGCGGATAAGAGAGCTGTTTTCTCA
 1000 1010 1020 1030 1040 1050 1060 1070 1080

AlaGluSerHisPheArgAsnSerMetProSerPheAlaIleSerGlyTyrGluValLeuPheLeuThrThrTyrAlaGlnAlaAlaAsn
 AGCAGAAAGTCATTTTCGTAATTCATGCTTCGTTTGAATTTCTGGATACGAGCTTCTATTCTAACAACATATGCACAAGCTGCCAA
 1090 1100 1110 1120 1130 1140 1150 1160 1170

ThrHisLeuPheLeuLeuLysAspAlaGlnIleTyrGlyGluGluTrpGlyTyrGluLysGluAspIleAlaGluPheTyrLysArgGln
 CACACATTTATTTTACTAAAGACGCTCAAATTTATGGAGAAGAATGGGATACGAAAAAGAATATTGCTGAATTTTATAAAACACA
 1180 1190 1200 1210 1220 1230 1240 1250 1260

LeuLysLeuThrGlnGluTyrThrAspHisCysValLysTrpTyrAsnValGlyLeuAspLysLeuArgGlySerSerTyrGluSerTrp
 ACTAAAACCTTACCCAAGAAATATACIGACCATTGTGTCAAATGGTATAATGTTGGATTAGATAAATTAAGAGGTTTCATCTTATGAATCTTG
 1270 1280 1290 1300 1310 1320 1330 1340 1350

ValAsnPheAsnArgTyrArgArgGluMetThrLeuThrValLeuAspLeuIleAlaLeuPheProLeuTyrAspValArgLeuTyrPro
 GGTAAACCTTTAACCGTTATCCGACAGAGATGACATTACAGTATTAGATTAAATTCGACTATITCCATTGTATGATGTTCCGCTATACCC
 1360 1370 1380 1390 1400 1410 1420 1430 1440

LysGluValLysThrGluLeuThrArgAspValLeuThrAspProIleValGlyValAsnAsnLeuArgGlyTyrGlyThrThrPheSer
 AAAAGAAGTTAAACCGAATTAACAGAGACGTTTTAACAGATCCAATTGTCCGAGTCAACAACCTTAGGGGCTATGGAACAACCTTCTC
 1450 1460 1470 1480 1490 1500 1510 1520 1530

AsnIleGluAsnTyrIleArgLysProHisLeuPheAspTyrLeuHisArgIleGlnPheHisThrArgPheGlnProGlyTyrTyrGly
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AsnAspSerPheAsnTyrTrpSerGlyAsnTyrValSerThrArgProSerIleGlySerAsnAspIleIleThrSerProPheTyrGly
 AAATGACTCTTTCAATTATTCGTCGCTAATTATGTTTCAACTAGACCAAGCATAGGATCAAAATGATATAATCACAATCTCCATTCTATGG
 1630 1640 1650 1660 1670 1680 1690 1700 1710

AsnLysSerSerGluProValGlnAsnLeuGluPheAsnGlyGluLysValTyrArgAlaValAlaAsnThrAsnLeuAlaValTrpPro
 AAATAAATCCAGTGAACCTGTACAAAATTTAGAAATTAATGGAGAAAAAGTCTATAGACCCGTAGCAAATACAAATCTTCGGGTCTGGCC
 1720 1730 1740 1750 1760 1770 1780 1790 1800

SerAlaValTyrSerGlyValThrLysValGluPheSerGlnTyrAsnAspGlnThrAspGluAlaSerThrGlnThrTyrAspSerLys
 GTCCGCTGTATATTCAGGTGTTACAAAAGTGAATTTAGCCAAATATAATGATCAAAACAGATGAAGCAAGTACACAAAAGTACGACTCAAA
 1810 1820 1830 1840 1850 1860 1870 1880 1890

ArgAsnValGlyAlaValSerTrpAspSerIleAspGlnLeuProProGluThrThrAspGluProLeuGluLysGlyTyrSerHisGln
 AAGAAATGTTGGCGCGTCAGCTGGGATTCTATCGATTCAATTGCCTCCAGAAACAACAGATGAACCTCTAGAAAAGGGATATAGCCATCA
 1900 1910 1920 1930 1940 1950 1960 1970 1980

LeuAsnTyrValMetCysPheLeuMetGlnGlySerArgGlyThrIleProValLeuThrTrpThrHisLysSerValAspPhePheAsa
 ACTCAATTATGTAATGTGCTTTTTAATGCAGGGTAGTAGGAACAATCCAGTGTAACTTGGACACATAAAAGTGTAGACTTTTTAA
 1990 2000 2010 2020 2030 2040 2050 2060 2070

MetIleAspSerLysLysIleThrGlnLeuProLeuValLysAlaTyrLysLeuGlnSerGlyAlaSerValValAlaGlyProArgPhe
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 2080 2090 2100 2110 2120 2130 2140 2150 2160

ThrGlyGlyAspIleIleGlnCysThrGluAsnGlySerAlaAlaThrIleTyrValThrProAspValSerTyrSerGlnLysTyrArg
 TACAGGACGAGATATCAITCAATGCACAGAAAAATGGAAGTCCGCAACTATTACGTTACACCGGATGTGTGCTACTCTCAAAAATATCG
 2170 2180 2190 2200 2210 2220 2230 2240 2250

AlaArgIleHisTyrAlaSerThrSerGlnIleThrPheThrLeuSerLeuAspGlyAlaProPheAsnGlnTyrTyrPheAspLysThr
 AGCTAGAATTCATTATGCTTCTACATCTCAGATAACATTTACACTCAGTTTAGACGGGGCAACATTTAATCAATACTATTTTCGATAAAAC
 2260 2270 2280 2290 2300 2310 2320 2330 2340

IleAsnLysGlyAspThrLeuThrTyrAsnSerPheAsnLeuAlaSerPheSerThrProPheGluLeuSerGlyAsnAsnLeuGlnIle
 GATAAATAAAGGAGACACATTAACTATAATTCAITTAATTTAGCAAGTTTCAGCACACCATTCGAATTATCAGGGAATACTTACAAT
 2350 2360 2370 2380 2390 2400 2410 2420 2430

GlyValThrGlyLeuSerAlaGlyAspLysValTyrIleAspLysIleGluPheIleProValAsn
 AGCGGTCACACGATTAAGTGGTGGAGATAAAGTTTATATAGACAAATTTGAATTTATCCAGTGAATTAATACTAGAAAGTAAAGAA
 2440 2450 2460 2470 2520

GTAGTCACCATCTATGATAGTAAGCAAAGGATAAAAAATGAGTTCATAAAATGAATAACATAGTGTTCITCAACTTTTCGCTTTTGAAG
 2530 2540 2550 2560 2570 2580 2590 2600 2610

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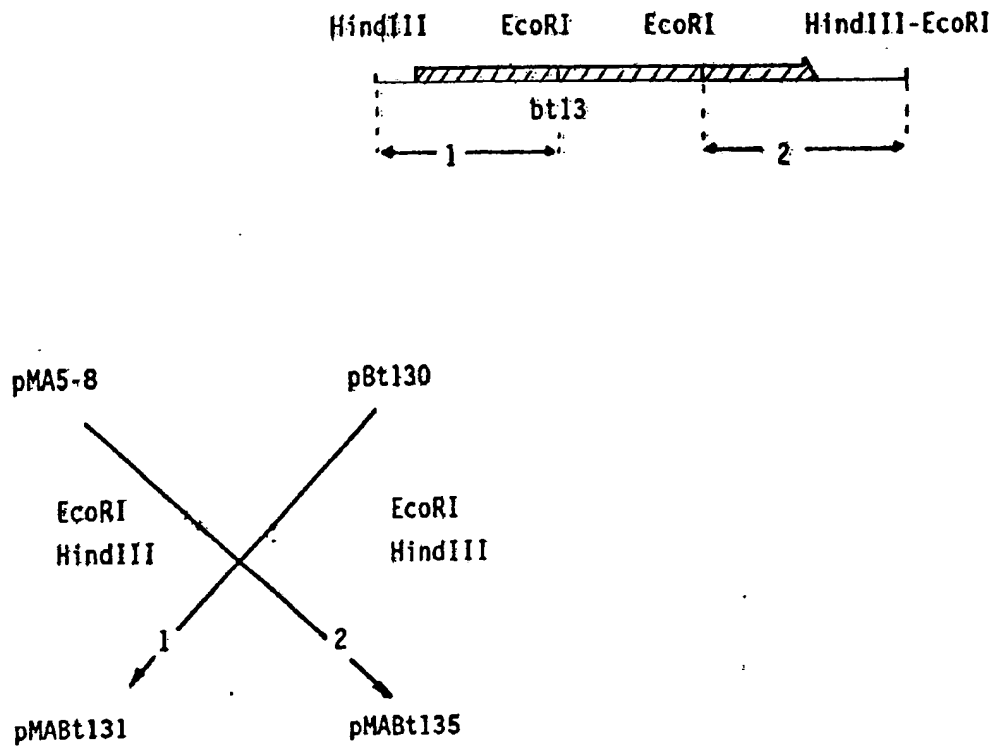
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TAGCTTCAAGACGCTTAGAATCATCAATATTTGTATACAGAGCTGTTGTTTTCATCGAGTTATGTCCCATTTGATTCGCTAATAGAACAA
 2800 2810 2820 2830 2840 2850 2860 2870 2880

GATCTTTATTTTCGTTATAATGATTGGTTGCATAAGTATGGCGTAATTTATGACGGCTTTTCTTTTCATCAAAAGCCCTCGTGTATTTCT
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CTGTAAGCTT
 2980

FIG. 4



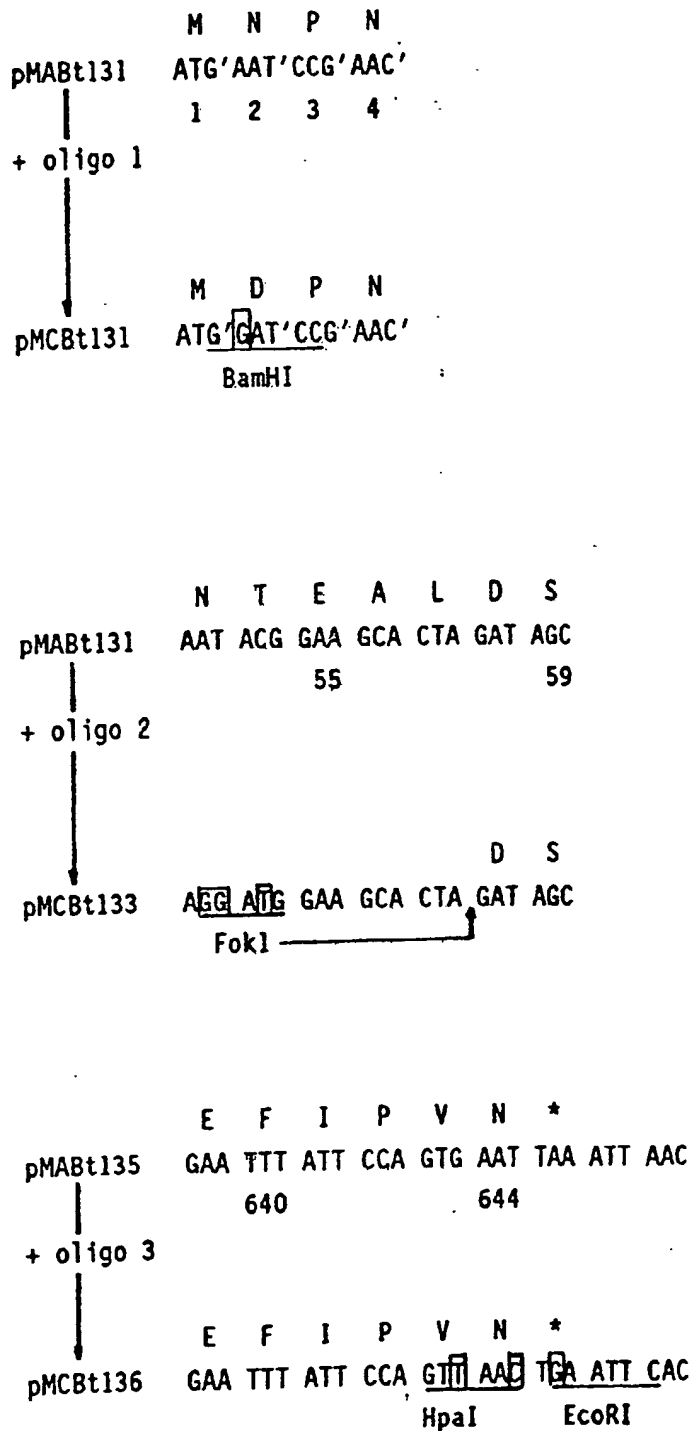


Fig. 5

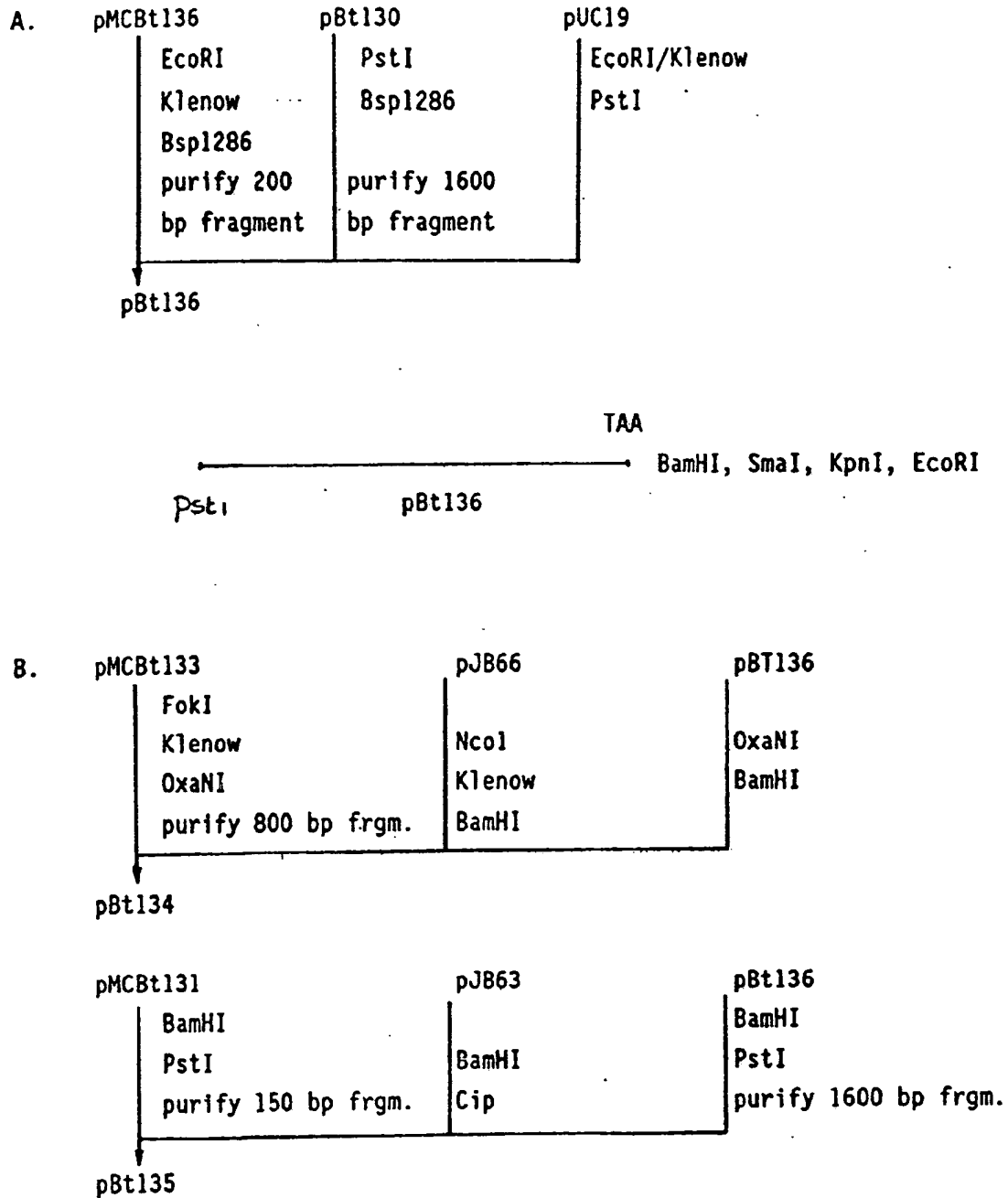
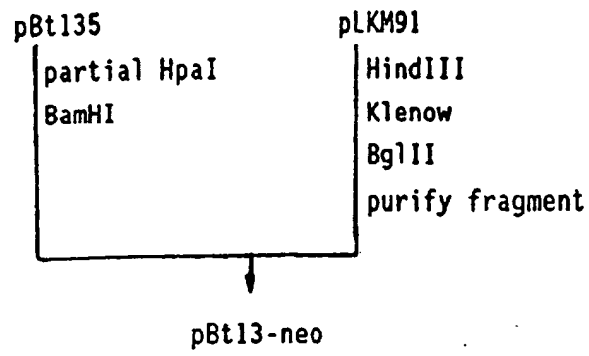


Fig. 6



643 5
---GTT,AGC,TTG,GAT,GGA
bt13 neo

Fig. 7

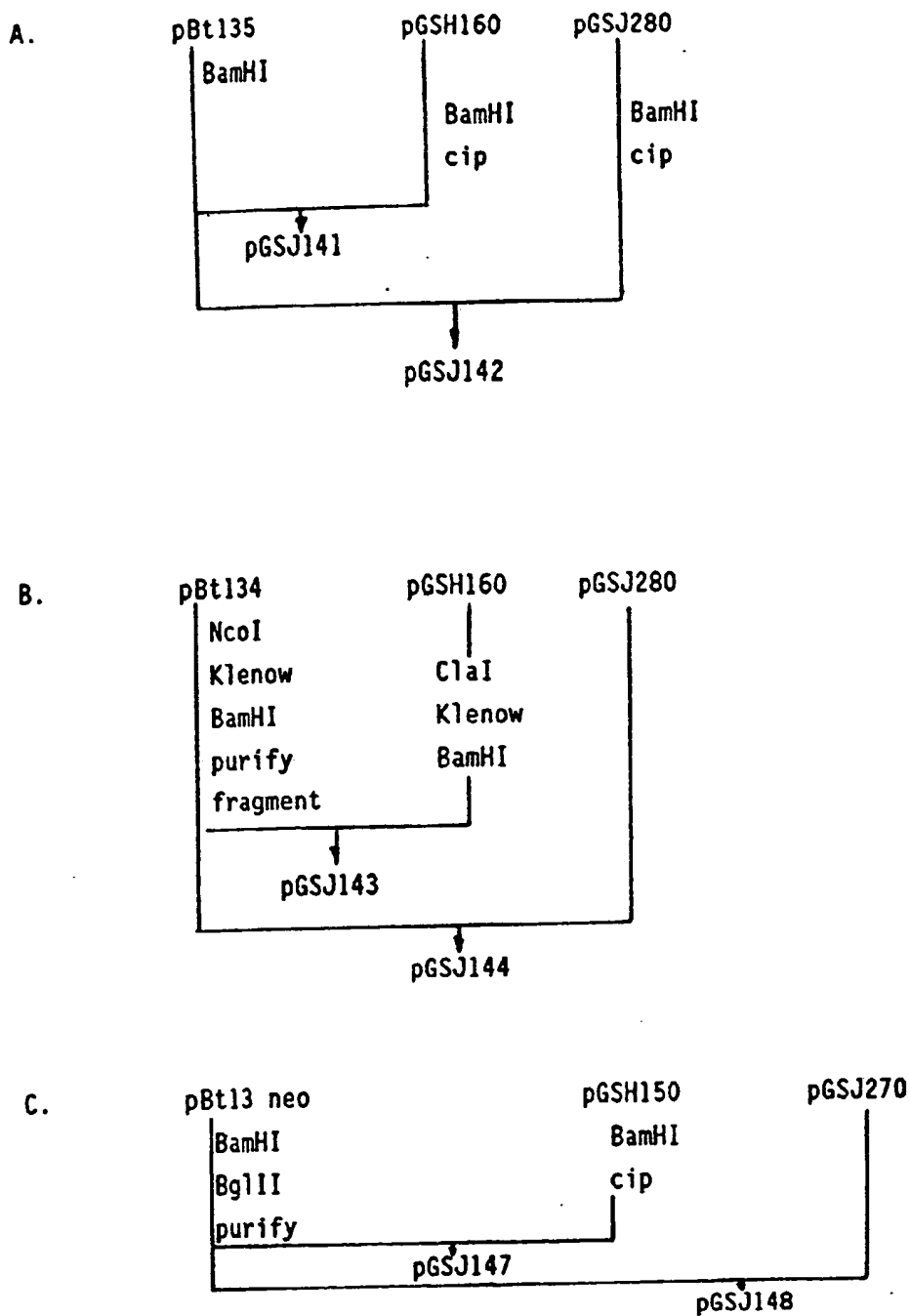


Fig. 8

D.

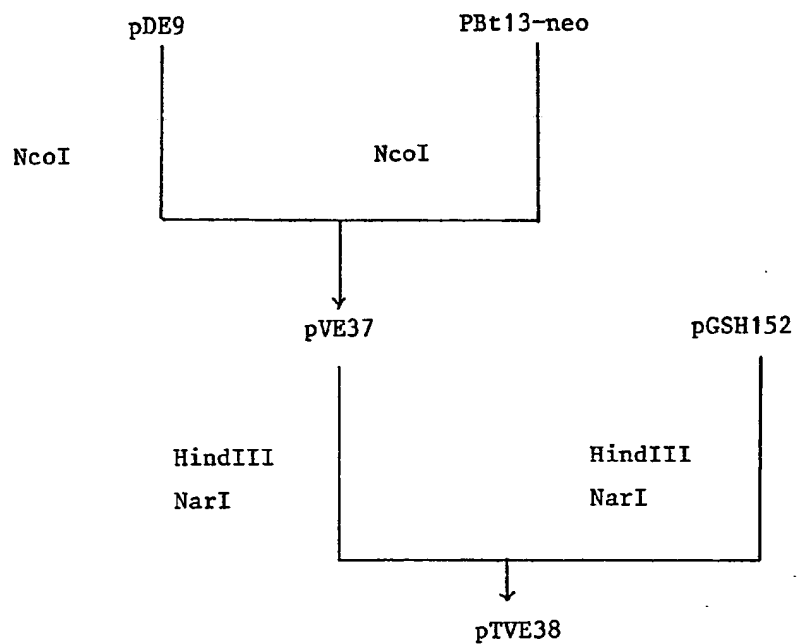


Fig 8

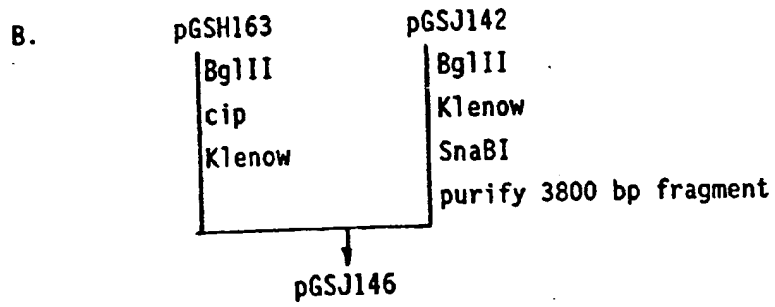
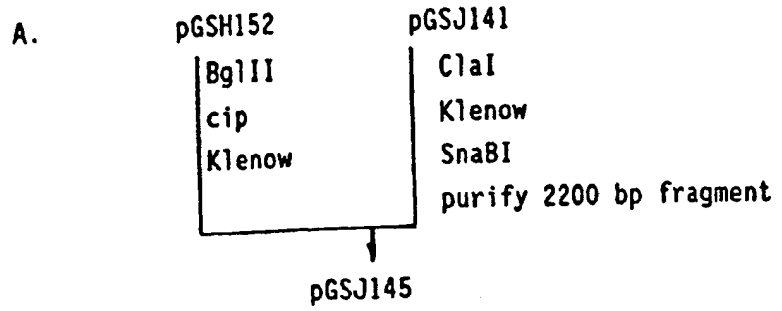


Fig. 9

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